

1 **Warming effect on nitrogen fixation in Mediterranean macrophyte sediments**

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17 **Running head:** Nitrogen fixation in Mediterranean macrophytes

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20 Warming, Thermal dependence, Activation Energy, Q₁₀, Mediterranean Sea.

21 **Abstract**

22 The Mediterranean Sea is warming faster than the global Ocean, with important
23 consequences for organisms and biogeochemical cycles. Warming is a major stressor for key
24 marine benthic macrophytes. However, the effect of warming on marine N₂ fixation remains
25 unknown, despite the high productivity of macrophytes in oligotrophic waters is partially
26 sustained by the input of new nitrogen (N) into the system by N₂ fixation. Here, we assess the
27 impact of warming on N₂ fixation rates of three key marine macrophytes: *Posidonia oceanica*,
28 *Cymodocea nodosa*, and *Caulerpa prolifera*. We experimentally measured N₂ fixation rates in
29 vegetated and bare sediments at temperatures encompassing current summer mean (25 and 27
30 °C), projected summer mean (29 and 31 °C) and projected summer maximum (33 °C) seawater
31 surface temperatures (SST) by the end of the century under a scenario of moderate greenhouse-
32 gas emissions. We found that N₂ fixation rates in vegetated sediments were 2.8-fold higher than
33 in bare sediments at current summer mean SST, with no differences among macrophytes.
34 Currently, the contribution of N₂ fixation to macrophytes productivity could account for up to 7
35 %, 13.8 % and 1.8 % of N requirements for *P. oceanica*, *C. nodosa*, and *C. prolifera*,
36 respectively. We showed the temperature dependence of sediment N₂ fixation rates. However,
37 the thermal response differed for vegetated sediments, where rates showed an optimum at 31 °C
38 followed by a sharp decrease at 33 °C, and bare sediments, where rates increased along the range
39 of the experimental temperatures. The activation energy and Q₁₀ were lower in vegetated than
40 bare sediments, indicating the lower thermal sensitivity of vegetated sediments. The projected
41 warming is expected to increase the contribution of N₂ fixation to Mediterranean macrophytes'
42 productivity. Therefore, the thermal dependence of N₂ fixation might have important
43 consequences for primary production in coastal ecosystems in the context of warming.

44 **1. Introduction**

45 Global mean surface temperatures have increased 0.85 °C from 1880 to 2012 and are
46 projected to increase between 1 and 3.5 °C by the end of the 21st century, relative to pre-
47 industrial times (IPCC, 2013). Similarly, heatwaves are more frequent since the second half of
48 the 20th century in Europe, Asia and Australia (IPCC, 2013; Perkins et al., 2012). Oceans store
49 most of the accumulated heat in the biosphere, warming at an average rate of 0.11 °C per decade
50 at surface (up to 75 m depth) since 1970 (IPCC, 2013), with longer and more frequent marine
51 heatwaves over the last century (Oliver et al., 2018). Warming is larger in small and enclosed
52 basins such as the Mediterranean Sea (Vargas-Yáñez et al., 2008), which is warming at 0.61 °C
53 per decade (Belkin, 2009), with rapid migration of marine isotherms (Burrows et al., 2011) and
54 increased thermal extremes (Diffenbaugh et al., 2007). Specifically, the maximum surface
55 seawater temperature (SST_{max}) in the Balearic Islands, in the western Mediterranean Sea, is
56 projected to increase by 3.4 ± 1.3 °C by 2100 under a scenario of moderate greenhouse-gas
57 (GHG) emissions (A1B scenario from the IPCC Special Report on Emissions Scenarios,
58 equivalent to the RCP6.0 scenario of the IPCC Fifth Assessment Report) (Jordà et al., 2012),
59 with important consequences for marine organisms and ecosystems.

60 Seagrass ecosystems provide important ecosystem services, such as the increase in
61 diversity, the reduction of wave action and the protection of coast, the increase in water clarity
62 by trapping suspended particles, and climate change mitigation by acting as carbon sinks
63 (Costanza et al., 1997; Duarte, 2017; Fourqurean et al., 2012). In the Mediterranean Sea, the most
64 relevant seagrass species are *Posidonia oceanica*, an endemic long-living seagrass, and
65 *Cymodocea nodosa*, commonly found in the Eastern Mediterranean Sea and the Northeastern
66 Atlantic Coast. Similarly, benthic green macroalgae, such as the autochthonous Mediterranean

67 *Caulerpa prolifera*, form highly productive ecosystems contributing to the atmospheric CO₂
68 sequestration (Duarte, 2017). However, these coastal vegetated ecosystems are threatened by
69 climate change at global scale (Duarte et al., 2018) and at the Mediterranean Sea scale (Marbà et
70 al., 2015). In particular, warming increases the mortality rates of *P. oceanica* (Marba and Duarte,
71 2010), which is predicted to be functional extinct by 2049 to 2100 due to warming (Chefaoui et
72 al., 2018;Jordà et al., 2012). Mesocosm experiments showed that *C. nodosa* is more resistant to
73 warming than *P. oceanica* (Olsen et al., 2012), concurrent with thermal niche models (Chefaoui
74 et al., 2016;Chefaoui et al., 2018); however a loss of 46.5 % in *C. nodosa* extension is predicted
75 by 2100 under the worst-case warming scenario (Chefaoui et al., 2018). Although *C. prolifera*
76 thrives well in warm waters, its photosynthesis is inhibited at temperatures above 30 °C (Lloret et
77 al., 2008;Vaquer-Sunyer and Duarte, 2013), compromising its survival at temperatures above this
78 threshold.

79 Warming also affects metabolic processes driving biogeochemical cycles in coastal
80 benthic ecosystems. Warming enhances sediment sulfate reduction rates (Robador et al., 2016),
81 leading to an increase in sulfide accumulation in coastal bare sediments (Sanz-Lázaro et al.,
82 2011) and seagrass colonized sediments (Koch et al., 2007). In the Mediterranean Sea,
83 heatwaves and warming trigger sulfide intrusion in *P. oceanica* shoots (García et al., 2013),
84 which has toxic effects on plant meristems (Garcias-Bonet et al., 2008) and increases shoot
85 mortality (Calleja et al., 2007). Sanz-Lázaro et al. (2011) found that warming enhances sediment
86 oxygen uptake and CO₂ emissions in coastal sediments, boosted by the addition of labile organic
87 matter, in a mesocosm experiment. Similarly, warming together with eutrophication have been
88 identified as main drivers of hypoxia in a Mediterranean macroalgae *Caulerpa prolifera* meadow

89 (Vaquer-Sunyer et al., 2012). However, studies on the effect of warming on atmospheric
90 nitrogen fixation in coastal marine ecosystems are lacking.

91 Nitrogen (N₂) fixation plays a fundamental role in balancing nutrient budgets at the basin
92 scale in the Mediterranean Sea, with most of this N₂ fixation associated with *P. oceanica*
93 seagrass meadows (Béthoux and Copin-Montégut, 1986). Indeed, endophytic nitrogen-fixing
94 bacteria have been detected in roots of *P. oceanica* (Garcias-Bonet et al., 2012;Garcias-Bonet et
95 al., 2016) and N₂ fixation has been reported in leaves and roots of *P. oceanica* (Agawin et al.,
96 2016;Lehnen et al., 2016) and *in situ* incubations (Agawin et al., 2017). Therefore, the high
97 productivity of *P. oceanica* in the oligotrophic Mediterranean waters is partially supported by N₂
98 fixation. However, the magnitude of N₂ fixation rates in the rhizosphere of *P. oceanica* is still
99 unknown, as well as N₂ fixation associated to other key macrophytes commonly found in the
100 Mediterranean Sea, such as *C. nodosa* and *C. prolifera*. In addition, whether warming will affect
101 N₂ fixation rates is still unknown.

102 Here, we test the hypothesis that N₂ fixation rates in coastal ecosystems is temperature
103 dependent and will increase with the forecasted warming. We do so by experimentally assessing
104 the response of N₂ fixation rates in coastal Mediterranean vegetated ecosystems to warming. We
105 focus specifically on the key macrophyte species most commonly found in the Mediterranean
106 Sea: two seagrass species (*P. oceanica*, *C. nodosa*) and one green macroalgae species (*C.*
107 *prolifera*). We experimentally measured N₂ fixation rates in vegetated and bare sediments at
108 temperatures encompassing current summer mean SST range (25 and 27 °C), projected summer
109 mean SST range (29 and 31 °C), and projected summer SST_{max} (33 °C) by the end of the century
110 under a scenario of moderate GHG emissions to assess: i) differences between vegetated and

111 bare sediments, ii) differences among macrophyte species and iii) the thermal dependence of
112 sediment N₂ fixation rates.

113

114 **2. Materials and methods**

115

116 **2.1. Study site**

117 The study was conducted with benthic communities sampled in Pollença Bay (Mallorca,
118 Spain), a bay located in the western Mediterranean Sea (39° 53.792' N; 3° 5.523' E). The study
119 site was selected based on the coexistence of the three most commonly found macrophyte
120 species in the region, including two seagrasses (*Posidonia oceanica* and *Cymodocea nodosa*) and
121 one green macroalgae (*Caulerpa prolifera*). The three macrophytes grow close to each other in
122 monospecific patches at 5-m depth. Mean (\pm SE) shoot density estimates were 699 ± 444 and
123 604 ± 136 shoot m⁻² for *P. oceanica* and *C. nodosa*, respectively (Marbà and Vaquer-Sunyer,
124 unpublished). The study was conducted in mid-June 2017 when *in situ* daily mean (\pm SE) SST
125 was 26.4 ± 0.08 °C.

126 We sampled sediment colonized by these three macrophytes and the adjacent bare
127 sediment using sediment cores (50 cm length and 4.5 cm in diameter). We collected 16 sediment
128 cores for each type of sediment. The vegetated sediment cores were collected from the center of
129 the macrophyte patches between shoots or blades, collecting belowground plant material but
130 avoiding the collection of aboveground biomass. The bare sediment cores were collected about 5
131 m away from the edge of the vegetated patches. We collected the sediment samples by pushing
132 the cores down into the sediment with the help of a rubber hammer and carefully extracting at
133 least 15 cm of undisturbed top sediment. The cores were transported immediately to the

134 laboratory. We measured seawater salinity using a calibrated conductivity meter (ProfiLine Cond
135 3310, WTW®, USA) and summer SST was monitored and recorded *in situ* every 2 hours from
136 2012 to 2017 using a Hobo logger (Onset Computer Corporation®, MA, USA). Full summer
137 SST records are available for 2012, 2013, 2106, and 2017. No data is available for 2015, and
138 only partial temporal coverage is available for 2014 (Fig. S1). The summer mean (\pm SE) SST
139 varied from 26.29 ± 0.05 °C in 2013 to 27.03 ± 0.04 °C in 2017, with an average summer mean
140 SST of 26.54 ± 0.17 °C from 2012 to 2017. Average summer minimum and maximum SST were
141 22.92 and 29.08 °C, respectively. The highest maximum SST was 29.67 °C and was registered in
142 August 2017 (Table S1).

143

144 **2.2. Nitrogen fixation rates**

145 We measured sediment N₂ fixation rates by the Acetylene Reduction Assay (Capone and
146 Taylor, 1980) in *P. oceanica*, *C. nodosa*, and *C. prolifera* vegetated sediments and the adjacent
147 bare sediment at five incubation temperatures: 25, 27, 29, 31, and 33 °C. The 25 and 27 °C
148 temperature treatments represent the current summer mean SST, covering the *in situ* recorded
149 average summer mean SST of 26.54 ± 0.2 °C (25 % percentile = 25.81 °C and 75 % percentile =
150 27.61 °C) from 2012 to 2017. The 29 and 31 °C temperature treatments represent the range of the
151 projected summer mean SST by the end of the century under a scenario of moderate GHG
152 emissions equivalent to RCP6.0, applying the projected mean SST increase of 2.8 ± 1.1 °C in the
153 region (Jordà et al., 2012) over the summer mean SST registered in 2017 (27.03 °C) . The 33 °C
154 temperature treatment represents the projected summer SST_{max} by the end of the century under a
155 scenario of moderate GHG emissions equivalent to RCP6.0, by applying the projected SST_{max}
156 increase of 3.4 ± 1.3 °C (Jordà et al., 2012) over the summer SST_{max} of 29.67 °C already

157 recorded in 2017. The sediment incubations were run in five water baths (i.e. one per
158 temperature treatment) equipped with thermometers and heaters located in a stable temperature
159 room. The target temperature for each water bath was maintained using an IKS-AQUASTAR
160 system which controlled and recorded the temperature every 10 minutes. During the incubations,
161 the temperature oscillation around the target temperatures ranged from 0.3 to 0.7 °C, and the
162 temperature accuracy was ± 0.05 °C on average (Table S2).

163 Once in the laboratory, the sediment from the cores was extruded carefully using a
164 plunger, and the first 10 cm of sediment below surface were collected, the rest of the sediment in
165 the core was discarded. For each replicate, 80 ml of sediment together with the belowground
166 biomass present was placed in a 500 ml glass bottle. No aboveground biomass was included in
167 the incubation glasses. Then, we added 200 ml of autoclaved seawater and the bottles were
168 closed with a lid fitted with a gas-tight valve. Finally, we added 20 ml of acetylene-saturated
169 seawater through the gas-tight valve of each bottle in order to achieve a final acetylene
170 concentration of 4 mM. The acetylene-saturated seawater was prepared according to Wilson et
171 al. (2012). We ran the sediment incubations in triplicate for each type of sediment and each
172 temperature treatment under dark conditions. The incubations lasted 24h, starting after the
173 addition of acetylene-saturated seawater. We sampled the headspace five times: at the start of the
174 experiment and at 12, 17, 20, and 24 h since the onset of the experiment. Specifically, we
175 withdrew 3 ml of air from the headspace with a gas-tight syringe. The headspace air sample was
176 immediately injected into a 3 ml vacuum vial for further analysis of ethylene concentration on a
177 gas chromatographer equipped with a flame ionization detector (FID-GC, Agilent 5890) using a
178 PoraPLOT U GC column (25 m \times 0.53 mm \times 20 μ m, Agilent Technologies, USA). We built a
179 calibration curve using three ethylene standards of known concentration (1.02, 10.13 and 99.7

180 ppm) and Helium as a balance gas, supplied by Carburos Metálicos S.A. (Palma de Mallorca,
181 Spain). We estimated the concentration of dissolved ethylene, from the ethylene concentration in
182 the equilibrated air as described previously (Wilson et al., 2012) and applying the solubility
183 coefficient of ethylene according to Breitbarth et al. (Breitbarth et al., 2004) as a function of
184 temperature and salinity.

185 We ran negative controls consisting in sediment without addition of acetylene-saturated
186 seawater in order to confirm that ethylene was not naturally produced by our samples, and
187 autoclaved seawater used in the preparation of the incubations with addition of acetylene-
188 saturated seawater in order to confirm that ethylene was not produced in the seawater. No
189 ethylene was produced in any the negative controls. The ethylene production rates were
190 converted into N₂ fixation rates by applying the common ratio of 3 mol of acetylene:1 mol of N₂
191 (Welsh, 2000).

192 At the end of the incubation, we dried the sediment samples at 60 °C and recorded the dry
193 weight for further calculations. Moreover, we calculated the sediment organic matter (OM)
194 content of each replicate sediment sample by loss on ignition (Dean Jr, 1974). The sediment N₂
195 fixation rates were first calculated by sediment dry weight and then standardized to surface area
196 integrated over 10 cm sediment depth by taking into account the sediment bulk density.

197

198 **2.3. Statistical analysis**

199 Differences in sediment OM content and bulk density among *P. oceanica*, *C. nodosa*, and
200 *C. prolifera* vegetated sediments and bare sediment were tested with the non-parametric Kruskal
201 Wallis test. Differences in sediment N₂ fixation rates among the four types of sediment (*P.*
202 *oceanica*, *C. nodosa*, and *C. prolifera* vegetated sediments and bare sediment) were tested by

203 Friedman test matching by temperature treatment. Then, we tested the effect of temperature (as a
204 categorical explanatory variable with 3 levels: current summer mean SST range (25 and 27 °C),
205 projected summer mean SST range (29 and 31 °C), and projected summer SST_{max} (33 °C)) and
206 type of sediment (as a categorical explanatory variable with 2 levels: vegetated and bare
207 sediments) on sediment N₂ fixation rates (our response variable) after a log transformation to
208 meet normality requirements by a full factorial two-way ANOVA. Finally, differences in
209 sediment N₂ fixation rates between vegetated and bare sediments were tested by non-parametric
210 Mann-Whitney *U* test at three different temperature ranges: current summer mean SST range (25
211 and 27 °C), projected summer mean SST range (29 and 31 °C), and projected summer SST_{max}
212 (33 °C). Moreover, we tested the thermal dependence of sediment N₂ fixation rates in vegetated
213 and bare sediments by fitting the Arrhenius function to estimate the activation energy (*Ea*),
214 derived from the linear regression between the natural logarithm of N₂ fixation rates and the
215 inverse of the temperature multiplied by the Boltzmann's constant (Dell et al., 2011), and Q₁₀,
216 the relative rate of increase in N₂ fixation expected for a 10°C temperature increase (Raven and
217 Geider, 1988). The Q₁₀ was calculated using the following equation (Raven and Geider, 1988):

$$218 \quad Q_{10} = e^{\left(\frac{10 Ea}{RT^2}\right)}$$

219 where R is the gas constant (8.314472 mol⁻¹K⁻¹), T is the mean absolute temperature across the
220 range over which Q₁₀ was measured (K), and *Ea* is the activation energy (J mol⁻¹). The activation
221 energy and Q₁₀ of N₂ fixation in vegetated sediments were calculated using the increasing rates
222 measured at four temperature treatments (25, 27, 29, and 31 °C), while the declining rates
223 measured at 33 °C were not included. The full range of temperature treatments was used for bare
224 sediments since no decline was detected. All statistical analyses were performed using JMP
225 (SAS Institute Inc., USA) and PRISM (GraphPad Software Inc., USA) statistical software.

226

227 3. Results

228 Sediment OM content was significantly different in the sediments colonized by different
229 macrophyte species ($\chi^2_{3,56} = 50.33$, $p < 0.0001$). *Posidonia oceanica* sediments had the highest
230 OM content (13.34 ± 0.56 %), whereas bare sediments had the lowest OM content (0.44 ± 0.50
231 %, Table 1). Sediment bulk density differed among sediment types ($\chi^2_{3,56} = 46.02$, $p < 0.0001$,
232 Table 1).

233 Average N_2 fixation rates in bare sediments was 0.06 ± 0.01 (range from 0.01 to 0.09)
234 $nmol\ N\ gDW^{-1}\ h^{-1}$; while, average N_2 fixation rates in vegetated sediments was three-fold greater
235 at 0.19 ± 0.03 (range from 0.05 to 0.9) $nmol\ N\ gDW^{-1}\ h^{-1}$, pooling all temperature treatments
236 together. Within the vegetated sediments, the maximum mean N_2 fixation rate was detected in *C.*
237 *prolifera* ($0.22 \pm 0.05\ nmol\ N\ gDW^{-1}\ h^{-1}$), whereas the minimum mean N_2 fixation rate was
238 measured in *C. nodosa* ($0.15 \pm 0.04\ nmol\ N\ gDW^{-1}\ h^{-1}$). Mean N_2 fixation rate in *P. oceanica*
239 was $0.21 \pm 0.06\ nmol\ N\ gDW^{-1}\ h^{-1}$. Nitrogen fixation rates differed among the four different
240 sediment types (i.e. bare, *P. oceanica*, *C. nodosa*, and *C. prolifera* sediments) ($\chi^2_{3,56} = 10.68$, $p =$
241 0.005) when expressed by sediment dry weight. However, once the rates were converted into
242 aerial basis, these differences were no longer significant ($\chi^2_{3,56} = 6.12$, $p > 0.05$), due to high
243 variability in sediment bulk densities. Sediment N_2 fixation rates were independent of OM
244 content (linear regression, $dfN = 1$, $dfD = 58$, Pearson's $r = 0.19$, $p > 0.05$).

245 Nitrogen fixation rates in aerial basis were significantly higher in vegetated sediments
246 compared to bare ones ($U = 154$, $p < 0.002$) when pooling all temperature treatments together,
247 with sediments colonized by macrophytes supporting, on average, twice the nitrogen fixation rate
248 than bare sediments (mean \pm SE = 3.86 ± 0.53 and $1.77 \pm 0.20\ mg\ N\ m^{-2}\ d^{-1}$, respectively),

249 considering all temperature treatments. Temperature and type of sediment (vegetated and bare
250 sediments) had a significant effect on N₂ fixation rates (two-way ANOVA; sediment type $F_{1,59} =$
251 10.40, $p < 0.01$; temperature $F_{2,59} = 4.89$, $p < 0.05$), with no significant interaction between
252 them. Specifically, at current summer SST range (25-27 °C), N₂ fixation rates in vegetated
253 sediments (3.15 ± 0.48 mg N m⁻² d⁻¹) were significantly higher ($U = 13$, $p < 0.01$) than those in
254 bare sediments (1.14 ± 0.3 mg N m⁻² d⁻¹) (Fig. 1A). Similarly, at projected summer mean SST
255 range (29-31 °C), N₂ fixation rates in vegetated sediments (5.25 ± 1.17 mg N m⁻² d⁻¹) were
256 significantly higher ($U = 23$, $p < 0.05$) than the rates measured in bare sediments (2.18 ± 0.2 mg
257 N m⁻² d⁻¹) (Fig. 1B). However, N₂ fixation rates did not differ between vegetated and bare
258 sediments at projected summer SST_{max} (33 °C), with N₂ fixation rates of 2.49 ± 0.248 and $2.21 \pm$
259 0.15 mg N m⁻² d⁻¹, respectively (Fig. 1C).

260 In vegetated sediments, N₂ fixation rates increased linearly with temperature up to 31°C
261 (N_2 fixation (nmol N g DW⁻¹ h⁻¹) = $-0.63 + 0.03 * \text{Temperature}$, $R^2 = 0.11$, $p < 0.05$), with a
262 marked decrease from 0.32 ± 0.09 nmol N g DW⁻¹ h⁻¹ at 31 °C to 0.11 ± 0.01 nmol N g DW⁻¹ h⁻¹
263 at 33 °C (Fig. 2). Nitrogen fixation rates in bare sediments increased linearly with temperature up
264 to 33 °C (N_2 fixation (nmol N g DW⁻¹ h⁻¹) = $-0.11 + 0.01 * \text{Temperature}$, $R^2 = 0.51$, $p < 0.005$, Fig.
265 2). The associated activation energies were 0.91 ± 0.39 and 1.25 ± 0.39 eV for vegetated and
266 bare sediments, respectively (Fig. 3). Associated Q₁₀ values were 3.84 ± 2.22 and 6.41 ± 2.97 for
267 vegetated and bare sediments, respectively.

268 4. Discussion

269 The overall average N₂ fixation rate found in Mediterranean vegetated sediments at
270 current summer mean SST (3.15 ± 0.48 mg N m⁻² d⁻¹) is within the range of those rates reported
271 for sediments colonized by temperate seagrass species (from 1.2 to 6.5 mg N m⁻² d⁻¹ in *Zostera*

272 *marina* sediments in the North Sea (McGlathery et al., 1998) and the Northwest Atlantic coast
273 (Capone, 1982), and from 0.1 to 7.3 mg N m⁻² d⁻¹ in *Zostera noltii* in the Northeast Atlantic coast
274 (Welsh et al., 1996)). However, N₂ fixation rates are lower than the rates reported for tropical
275 and subtropical seagrass species (see references in Welsh, 2000). The overall N₂ fixation rates in
276 vegetated sediments are higher than in bare sediments, consistent with the long-recognized role
277 of marine plants in enhancing N₂ fixation rates (Capone, 1988). Specifically, the vegetated
278 sediments supported 3 to 4-fold higher N₂ fixation rates than bare sediments did at current
279 summer mean SST range when expressed by area and by sediment dry weight, respectively.

280 The N₂ fixation rates we measured in *P. oceanica* sediments at current summer mean
281 SST (2.86 ± 1.26 mg N m⁻² d⁻¹) are higher than the rates reported in summer, at similar seawater
282 temperature, by Agawin et al. (2017) using benthic bell-jar chambers containing *P. oceanica*
283 shoots and the underlying sediment (ranging from 0.06 to 1.51 mg N m⁻² d⁻¹). However, the
284 different methodological approaches make comparisons difficult: while sediment slurries might
285 slightly overestimate rates due to sediment structure disturbance and increase in organic matter
286 availability, incubation chambers might underestimate rates due to poor diffusion of acetylene
287 into the sediment (Welsh, 2000). Nevertheless, the N₂ fixation rates in bare sediments at current
288 summer mean SST (1.14 ± 0.31 mg N m⁻² d⁻¹) are very similar to those measured by benthic bell-
289 jar in bare sediment adjacent to a *P. oceanica* meadow (from 0.01 to 1.99 mg N m⁻² d⁻¹) (Agawin
290 et al., 2017), suggesting that these differences in N₂ fixation rates in *P. oceanica* sediment might
291 also be due to variability among sites. The N₂ fixation rates in *C. nodosa* and *C. prolifera*
292 sediments reported here are the first reports, to the best of our knowledge, for these two
293 important Mediterranean macrophyte species. Indeed, the analysis of sediment N stocks in a *C.*
294 *nodosa* meadow in the Mediterranean Sea suggested that N₂ fixation might be contributing to

295 enhance the N stocks compared to bare sediments (Pedersen et al., 1997). The similar stable N
296 isotope composition of *C. nodosa* tissues and those of *P. oceanica* in the Mediterranean
297 (Fourqurean et al., 2007) also suggests that they use similar sources of nitrogen. The N₂ fixation
298 rates at current summer mean SST in *C. prolifera* sediments found here (0.17 ± 0.04 nmol N
299 gDW⁻¹ h⁻¹) are similar to the sediment N₂ fixation rates associated to the invasive *C. taxifolia* in
300 Monaco (0.12 ± 0.09 nmol N gDW⁻¹ h⁻¹) but 20-fold lower than the N₂ fixation rates reported for
301 *C. taxifolia* in France (3.96 ± 1.99 nmol N gDW⁻¹ h⁻¹) (Chisholm and Moulin, 2003).

302 Although the sediments colonized by these three macrophyte species hold similar rates,
303 the contribution of sediment N₂ fixation to the productivity of each plant is different. Taking into
304 account the average net production (2.63 and 1.47 g DW m⁻² d⁻¹ for *P. oceanica* and *C. nodosa*,
305 respectively (Duarte and Chiscano, 1999) and 5.16 g DW m⁻² d⁻¹ for *C. prolifera* (Marbà
306 unpublished)) and the tissue nitrogen content (from 1.55 to 1.63 % for *P. oceanica*, from 1.91 to
307 2.28 % for *C. nodosa* (Duarte, 1990;Fourqurean et al., 2007) and from 3 to 4.9 % for *C. prolifera*
308 (Morris et al., 2009)), the mean measured sediment N₂ fixation rates detected at current summer
309 mean SST (25 and 27 °C) could account for 6.7 to 7 %, 11.5 to 13.8 % and 1.1 to 1.8 % of the
310 nitrogen requirements for *P. oceanica*, *C. nodosa*, and *C. prolifera*, respectively. The calculated
311 contribution of N₂ fixation to seagrass growth requirements fall within the range of the N₂
312 fixation contributions reported for temperate seagrasses, ranging from 5 to 12 % for *Z. marina*
313 and *Z. noltii*, respectively (Welsh, 2000). The calculated contribution of N₂ fixation to fulfill the
314 macrophytes' growth requirements points N₂ fixation as partially supporting the high
315 productivity of these primary producers in the Mediterranean oligotrophic waters.

316 We experimentally demonstrate that N₂ fixation in coastal sediments is thermal
317 dependent, both in vegetated and bare sediments. Despite a formal experimental demonstration

318 was lacking, the N₂ fixation thermal dependence reported here is in agreement with the higher
319 rates typically measured in warm tropical and subtropical meadows compared to the rates
320 reported in temperate and cold seagrass systems (Herbert, 1999;McGlathery, 2008;Welsh, 2000).
321 The thermal dependence, as reflected by the activation energy and Q₁₀, for N₂ fixation rates was,
322 however, higher in bare sediments than in vegetated sediments, possibly due to different
323 bacterial communities. Westrich and Berner (1988) also found that sulfate reduction exhibited a
324 more pronounced thermal dependence in sediments supporting lower rates. The activation
325 energies for N₂ fixation in vegetated sediments (0.91 ± 0.4 eV or 87.8 ± 37.6 KJ mol⁻¹) and in
326 bare sediments (1.25 ± 0.4 eV or 120.6 ± 37.6 KJ mol⁻¹) are within the range of the activation
327 energy reported for sediment sulfate reduction (range from 36 to 132 KJ mol⁻¹ (Robador et al.,
328 2016;Westrich and Berner, 1988)) and for sediment organic matter degradation (range from 54 to
329 125 KJ mol⁻¹ (Middelburg et al., 1996)). The Q₁₀ values associated to sediment N₂ fixation (3.84
330 ± 2.22 and 6.41 ± 2.97 for vegetated and bare sediments, respectively) are higher than those
331 reported for sediment sulfate reduction (from 1.6 to 3.4 (Robador et al., 2016)), but still similar
332 to those values associated to organic matter degradation (from 2.2 to 6.3 (Middelburg et al.,
333 1996)). Moreover, the thermal response differed for vegetated sediments, where N₂ fixation rates
334 showed an optimum at 31 °C followed by a sharp decrease at 33 °C, and bare sediments, where
335 N₂ fixation rates increased along the range of experimental temperatures tested here. The thermal
336 response of N₂ fixation in vegetated sediments found here is similar to the thermal response
337 reported for N₂ fixation in soil crusts (Zhou et al., 2016) and seagrass rhizosphere (Garcias-Bonet
338 et al., 2018), with an increase in rates up to 30 and 29 °C, respectively, and a marked decrease in
339 rates at temperatures above the optimum. The forecasted warming by the end of the century
340 could potentially increase N₂ fixation rates by 36.7% in vegetated sediments and 46.8% in bare

341 sediments. However, the decrease in N₂ fixation rates in vegetated sediments at 33 °C would
342 imply a reduction of a third in the contribution of N₂ fixation to the macrophytes' productivity
343 during heatwaves. The forecasted warming could affect as well other biogeochemical processes
344 in coastal sediments, such as sulfate reduction (Robador et al., 2016), anaerobic ammonium
345 oxidation and denitrification (Garcias-Bonet et al., 2018; Nowicki, 1994), among others, and,
346 therefore, potential synergic or antagonistic effects may occur. Responses of sediments colonized
347 by different macrophyte species may also differ due to differences in the lability of their OM and
348 nutrient stocks, associated to differences in C:N:P ratios (Enríquez et al., 1993; Lanari et al.,
349 2018). Similarly, losses of seagrass coverage by heatwaves could lead to an increase in CO₂
350 emissions by increased remineralization of C stocks (Arias-Ortiz et al., 2018).

351 The thermal dependence of N₂ fixation in vegetated sediments found here might have
352 important consequences for primary production in coastal ecosystems in the context of warming.
353 This may not be the case for *P. oceanica*, as this species is projected to be critically
354 compromised, to the extent that functional extinction is possible, with projected Mediterranean
355 warming rates by 2050-2100 (Chefaoui et al., 2018; Jordà et al., 2012). However, in order to
356 draw general conclusions on the effect of warming on N₂ fixation in coastal ecosystems, the
357 thermal-dependence found here needs be tested for a diversity of seagrass ecosystems. Similarly,
358 our results from experimental temperature treatments did not account for potential acclimation
359 and adaptation of microbial communities to warming, which should also be tested. Moreover, N₂
360 fixation is likely to be subjected to other environmental controls that may change, either in an
361 additive, synergistic or antagonistic manner, with warming, so predicting N₂ fixation rates in a
362 future, warmer coastal ocean remains challenging.

363

364 **Conclusion**

365 Mediterranean macrophyte meadows are sites of intense N₂ fixation rates, twice as high
366 as those in adjacent bare sediments. As these rates increased with warming, realized warming of
367 the Mediterranean Sea is expected to have led to enhanced sediment N₂ fixation rates, with future
368 warming leading to further increase in N₂ fixation rates up to 33 °C in bare sediments and 31 °C
369 followed by a decrease at higher temperatures in vegetated sediments. However, more work
370 covering a larger area is needed to confirm a generalized warming effect on sediment N₂
371 fixation.

372

373 **Author contribution**

374 NG-B, RVS, CMD and NM designed the study. NG-B and RVS performed the field
375 work, and sample and data analysis. NG-B, RVS, CMD and NM interpreted the results. NG-B
376 wrote the first draft of the manuscript. All authors contributed substantially to the final
377 manuscript.

378

379 **Conflict of interests**

380 Authors declare that they have no conflict of interest

381

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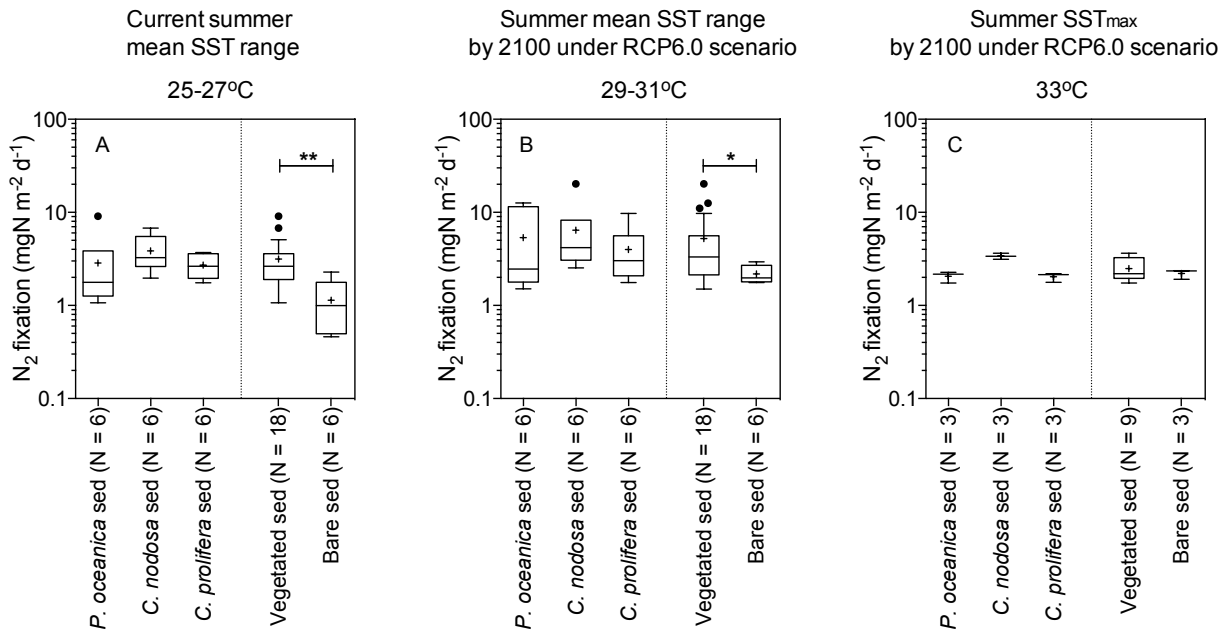
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388 **Figures**

389 Fig. 1. Box plot of the N₂ fixation rates (expressed by area) of sediments colonized by different
 390 macrophytes (as well as grouping all vegetated sediments together) and bare sediment measured
 391 at current summer SST range (25-27 °C, (A)), projected summer mean SST range by 2100 under
 392 RCP6.0 scenario (29-31 °C, (B)), and projected summer SST_{max} by 2100 under RCP6.0 (33 °C,
 393 (C)). Boxes extend from 25th to 75th percentiles, whiskers are calculated using the inter-quartile
 394 distance (IQR) according to the Tukey method, lines inside boxes represents the median, “+”
 395 represents the mean, and dots represent individual values greater than the 75th percentile plus 1.5
 396 X IQR. Statistically significant differences are indicated by asterisks where * indicates p < 0.05,
 397 and ** indicates p < 0.01. The sample size (N) is also indicated.

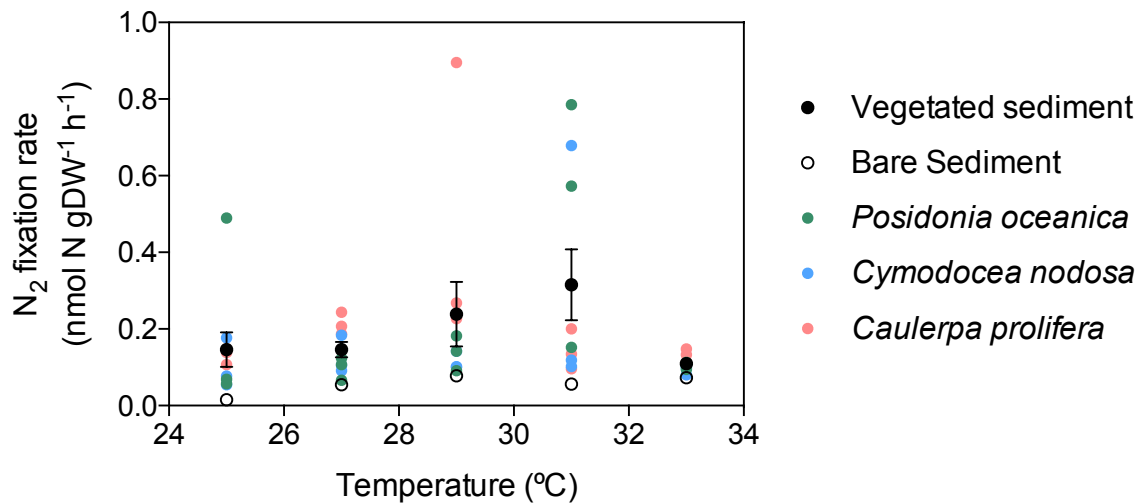
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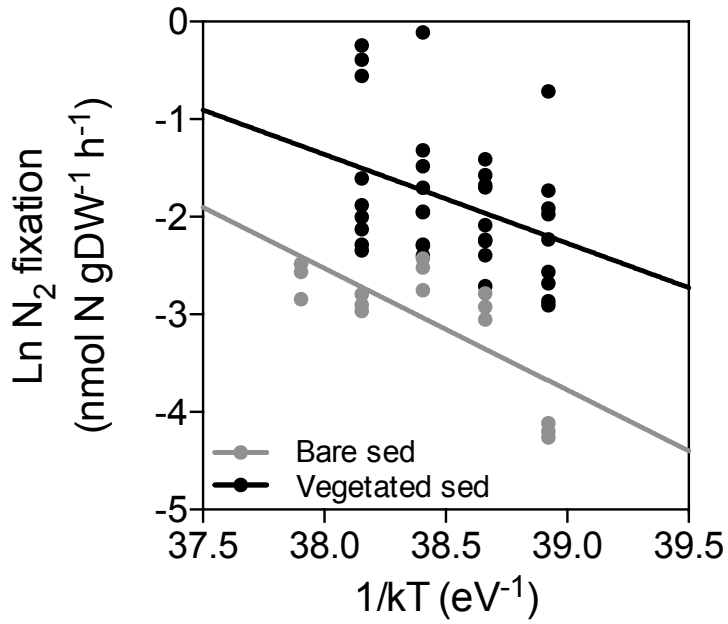
401 Fig. 2. Relationship of experimental incubation temperature and mean sediment N₂ fixation rates
402 (expressed by sediment dry weight) in vegetated (black dots) and bare sediments (clear dots).
403 Black and clear dots indicate mean values, and errors bars indicate standard error of the mean.
404 Individual replicate measurements of N₂ fixation rates for each macrophyte species are also
405 shown in colored coded dots, where green dots represent measurements on *P. oceanica*
406 sediments, blue dots represent measurements on *C. nodosa* sediments, and pink dots represent
407 measurements on *C. prolifera* sediments.
408



409
410
411

412 Fig. 3. Arrhenius plot for N₂ fixation rates in vegetated (black dots) and bare sediments (grey
413 dots), showing the linear regression between ln N₂ fixation rates and the inverse of the
414 temperature multiplied by the Boltzmann's constant (1/kT) for vegetated (black solid line) and
415 bare (grey solid line) sediments.

416



420 **Tables**

421

422 Table 1. Organic matter content and bulk density in sediments colonized by different macrophyte
 423 species and bare sediment in Pollença Bay (Mallorca) in June 2017. Mean values (\pm SEM), the
 424 ranges (minimum – maximum values), and the sample size (N) are shown.

425

| | <i>P. oceanica</i> sediment | <i>C. nodosa</i> sediment | <i>C. prolifera</i> sediment | Bare sediment |
|---|--|---|--|--|
| Sediment organic matter content (%) | 12.58 \pm 0.95 (8.17 – 20.63) N = 15 | 6.14 \pm 0.39 (4.49 – 10.22) N = 15 | 0.74 \pm 0.12 (0.04 – 1.31) N = 15 | 0.44 \pm 0.08 (0.02 – 1.41) N = 15 |
| Sediment bulk density (g DW sed cm ⁻³) | 0.54 \pm 0.03 (0.29 – 0.69) N = 15 | 1.01 \pm 0.03 (0.74 – 1.24) N = 15 | 0.46 \pm 0.02 (0.32 – 0.56) N = 15 | 0.96 \pm 0.02 (0.84 – 1.09) N = 15 |

426

427

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